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Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 8-13, 56 and 70-75 are pending in the application, with claim 8 being the sole independent claim. Claim 8 is sought to be amended. The amendment to claim 8 has been made to make explicit the subject matter that was originally intended to be encompassed by the claim. The amendment to claim 8 therefore does not introduce any new matter. It is believed that the amendment presented above will place the application in condition for allowance and/or in better form for appeal. *See* 37 C.F.R. § 1.116(a). It is respectfully requested that the amendment after final Office Action be entered and considered.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

I. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 8-13, 56 and 70-75 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. *See* Office Action at page 2. The rejection is based on the expression "incubating said mixture under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of said double-stranded DNA and sufficient to degrade single-stranded RNA," which was found in claim 8 prior to the

amendment filed on December 11, 2003. In the December 11, 2003 amendment, the phrase in question was changed to "incubating said mixture under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of said double-stranded DNA and under which said peptides or polypeptides having ribonuclease activity degrade single-stranded RNA." The Examiner has maintained the rejection on the ground that the amendment "has not resulted in the addition of an 'active RNA degradation step'." *See* Office Action at page 3.

Although Applicants respectfully disagree with the rationale underlying this rejection, Applicants request that claim 8 be further amended to recite ". . . thereby degrading single-stranded RNA in said preparation." Accordingly, claim 8, as currently presented, recites an "active RNA degradation step." Applicants believe that the rejection under 35 U.S.C. § 112, second paragraph, has been fully accommodated and should be withdrawn.

II. Claim Rejections Under 35 U.S.C. § 102

A. Davey

Claims 8, 9, 10, 13 and 71-73 were rejected under 35 U.S.C. § 102(b) as being anticipated by Davey *et al.* (U.S. Patent No. 5,409,818) ("Davey"). *See* Office Action at page 4. Applicants respectfully traverse this rejection.

An anticipation rejection under 35 USC § 102 requires a showing that each limitation of a claim is found in a single reference, practice, or device. *See In re Donohue*, 766 F.2d

531, 226 USPQ 619, 621 (Fed. Cir. 1985). The present claims are directed to methods for synthesizing a nucleic acid molecule from a preparation comprising RNA and double-stranded DNA. The methods comprise: (a) mixing the preparation with one or more DNA polymerases, and one or more peptides or polypeptides having ribonuclease activity; and (b) incubating the mixture under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of the double-stranded DNA and under which the peptides or polypeptides having ribonuclease activity degrade single-stranded RNA, thereby degrading single-stranded RNA in the preparation.

Davey does not describe a method comprising incubating a mixture comprising RNA, double-stranded DNA, one or more DNA polymerases, and one or more peptides or polypeptides having ribonuclease activity under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of the double-stranded DNA and under which the peptides or polypeptides having ribonuclease activity degrade single-stranded RNA, *thereby degrading single-stranded RNA in the preparation*. Therefore Davey cannot and does not anticipate the currently presented claims. Applicants respectfully request that this rejection be reconsidered and withdrawn.

B. Kenten

Claims 8, 9, 10, 13 and 72 were rejected under 35 U.S.C. § 102(e) as being anticipated by Kenten *et al.* (U.S. Patent No. 6,048,687) ("Kenten"). *See* Office Action at page 5. Applicants respectfully traverse this rejection.

Kenten does not describe a method comprising incubating a mixture comprising RNA, double-stranded DNA, one or more DNA polymerases, and one or more peptides or polypeptides having ribonuclease activity under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of the double-stranded DNA and under which the peptides or polypeptides having ribonuclease activity degrade single-stranded RNA, *thereby degrading single-stranded RNA in the preparation*. Therefore Kenten cannot and does not anticipate the currently presented claims. Applicants respectfully request that this rejection be reconsidered and withdrawn.

III. Claim Rejections Under 35 U.S.C. § 103

A. Davey

Claims 70, 74 and 75 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Davey. *See* Office Action at page 6. Applicants respectfully traverse this rejection.

A *prima facie* case of obviousness cannot be established unless all of the claim elements are taught or suggested by the cited references. *See In re Royka*, 490 F.2d 981, 984-85 (CCPA 1974); *see also In re Glaug*, 283 F.3d 1335, 1341-42 (Fed. Cir. 2002); *In re Rijckaert*, 9 F.3d 1531, 1533 (Fed. Cir. 1993). Davey does not teach or suggest a method comprising incubating a mixture comprising RNA, double-stranded DNA, one or more DNA polymerases, and one or more peptides or polypeptides having ribonuclease activity under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of the double-stranded DNA and under which the peptides or polypeptides having

ribonuclease activity degrade single-stranded RNA, thereby degrading single-stranded RNA in the preparation.

Since Davey does not teach or suggest all of the elements of the currently presented claims, a *prima facie* case of obviousness has not been established. Applicants respectfully request that this rejection be reconsidered and withdrawn.

B. Major and Maudru

Claims 8-12, 70, 71 and 73 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Major, *Biotechniques 12*:40-43 (1992) ("Major") and Maudru *et al.*, *J. Virol. Methods. 66*:247-261 (1997) ("Maudru"). *See* Office Action at page 7. Applicants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine reference teachings. *See In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998). The teaching or suggestion to make the claimed combination must be found in the prior art, not in Applicants' disclosure. *See In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). As explained below, a person of ordinary skill in the art would not have been motivated to combine or modify the teachings of Major and/or Maudru. Therefore, a *prima facie* case of obviousness based on these references cannot be established.

Major relates to a PCR-based method for screening for point mutations. The method of Major involves the use of oligonucleotide primers, the 3'-terminal nucleotide of which is

non-complementary to a specific nucleotide in a target sequence ("3'-terminal mismatch"). *See* Major at page 40, Figure 1. The principle behind this method is that PCR amplification should not occur when the 3'-terminal nucleotide of a primer does not base-pair with the corresponding nucleotide on the template nucleic acid. *See* Major at page 42, left column. It is noted in Major that, in certain cases, PCR amplification will occur despite the presence of a 3'-terminal nucleotide mismatch. *See* Major at page 42, center column (citing Wu *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2757-2760 (1989) and Kwok *et al.*, *Nucl. Acids Res. 18*:999-1005 (1990)). According to Major, "Even bacterial colony lysates showed clear negative results with all three 3'-terminal mismatches; however T:T mismatches gave some extra minor bands." *See* Major at page 42, bottom center column. The "extra minor bands" are amplification products produced using oligonucleotides that have 3'-terminal nucleotide mismatches (which, ideally, should not be extended in an amplification reaction in order to identify point mutations).

The obviousness rejection is based on the Examiner's position that a person of ordinary skill in the art would have somehow attributed the "extra minor bands" mentioned in Major to the presence of RNA in the reactions. According to the Examiner:

> One of ordinary skill in the art at the time of filing would have been motivated to add a polypeptide with ribonuclease activity to the method taught by Major, in order to remove residual RNA sequence contamination from the targeted nucleic acid template in any preparation which would contain substantial amounts of RNA, such as bacterial colony lysate, in order to decrease the level of background signal from the taught PCR assay.

See Office Action dated February 11, 2003 at page 8. Applicants respectfully disagree with this position. As pointed out by Applicants in their previous replies and discussed in more detail below, a person of ordinary skill in the art would not have considered RNA to be a factor responsible for the production of the "extra minor bands" mentioned in Major. *See, e.g.*, Applicants' Amendment and Reply Under 37 C.F.R. § 1.111, filed on December 11, 2003, at page 16.

In response to Applicants remarks set forth in their previous replies, the Examiner stated that:

> While Major does not attribute background difficulties to contaminating RNA, one of skill in the art would realize that given the employment of the method of Major to bacterial lysates, there would be a substantial amount of background RNA in the preparation. This knowledge in combination with that taught by Maudru et al. stating that the background signal in a similar assay was found to be due to an intrinsic RNA-dependent DNA polymerase activity of the *Taq* DNA polymerase would lead one of skill in the art who was attempting to successfully use a PCR method to screen for small mutations to include a ribonuclease digestion step prior to PCR amplification as a means of making the assay more sensitive. In support of the above, applicants attention is drawn to Major, page 42, middle column, which states "the present results indicate that all three possible terminal T mismatches can be equally discriminated under standard PCR conditions, **especially** when using mini-prep DNA". Such a statement clearly supports that even Major recognized the taught method had different results or sensitivities depending on the template used (noting the reference to "especially"), although Major did not comment on the specific difference of the two different types of template preparations. One of skill in the art would understand that the difference was likely the presence of contaminating material, such as RNA.

Office Action at pages 8-9 (emphasis in original). Applicants respectfully disagree with these assertions.

The logic underlying the rejection, as set forth by the Examiner, can be summarized as follows: (1) a person of ordinary skill in the art would have recognized that bacterial colony lysates contain "a substantial amount of RNA;" (2) Major indicates that better discrimination of terminal T mismatches was obtained using mini-prep DNA than with bacterial colony lysates; therefore, (3) a person of ordinary skill in the art would have attributed the difference in terminal T mismatch discrimination to the presence of RNA in bacterial colony lysates. This reasoning is both logically and technically flawed and cannot support a *prima facie* case of obviousness.

First, a person of ordinary skill in the art would have appreciated that bacterial colony lysates contain many factors besides RNA (*e.g.*, proteins, salts, lipids, signaling molecules, etc.), and that, like RNA, these other factors are absent from mini-prep DNA. The Examiner has not explained why a person of ordinary skill in the art, considering all the factors found in bacterial colony lysates, would have specifically regarded RNA as the one factor responsible the difference in 3'-terminal T mismatch discrimination alluded to in Major.

There has been no evidence presented to indicate that, at the time of the effective filing date of the present application, persons of ordinary skill in the art would have regarded RNA as a factor which might interfere with nucleic acid synthesis reactions. In fact, Applicants note that the only document of record that indicates that RNA can interfere with nucleic acid synthesis is *Applicants' own specification*. *See, e.g.*, specification at page 2,

lines 20-23. Applicants' own specification, however, cannot be used to establish a *prima facie* case of obviousness. *See In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). The Examiner has not pointed to any evidence to support the contention that, despite the large number and variety of factors that are found in bacterial colony lysates and not in mini-prep DNA, a person of ordinary skill in the art would have believed that RNA was the specific factor which caused the difference in terminal T mismatch discrimination mentioned in Major. Since this contention is the basis of the obviousness rejection and there is no evidence to support it, it follows that a *prima facie* case of obviousness has not been established.

The second flaw in the rejection is the implication that a person of ordinary skill in the art would have believed that errors in terminal nucleotide mismatch discrimination occur only in bacterial colony lysates (which contain RNA) but not in other DNA preparations (which do not contain RNA). To the contrary, it was well known in the art at the time of the effective filing date of the present application that errors in terminal mismatch discrimination (*i.e.*, extension of oligonucleotides with mismatched 3'-terminal nucleotides) occurred in purified DNA preparations.

For example, Kwok *et al.*, *Nucl. Acids Res. 18*:999-1005 (1990) ("Kwok") (copy submitted herewith as Exhibit 1), describe an assay system involving the use of either plasmid DNA or PCR-generated products as templates for PCR extension reactions. *See* Kwok, page 1001, left column. Using these samples -- *which lacked RNA* -- template amplification was observed using several primers that had mismatched 3'-terminal nucleotides. *See, e.g.*, Kwok at page 1001, right column ("the presence of a T at the 3' end

of the primer provided efficient amplification irrespective of the corresponding nucleotide in the template.") Kwok attributed these errors in terminal mismatch discrimination to factors such as dNTP concentration and primer length. *See* Kwok at page 1004, right column. (Kwok was cited in Major as reference No. 6.) Clearly, RNA could not have been a factor for the terminal mismatch discrimination errors described in Kwok.

Since it was well known in the art that errors in terminal mismatch discrimination occurred in samples that did not contain any RNA, a person of ordinary skill in the art would have appreciated that other factors, aside from RNA, were responsible for errors in terminal mismatch discrimination. A discussion of various factors that influence 3'-terminal mismatch discrimination is found in Charlieu, "Chapter 12, Distinction Between Almost-Identical DNA Sequences by Polymerase Chain Reaction," in *PCR Technology Current Innovations*, pp. 101-106, Griffin and Griffin *Eds.*, (1994) (copy submitted herewith as Exhibit 2). Factors mentioned in Charlieu include the nature of the DNA template, the nucleotide concentration, $MgCl_2$ concentration, *Taq* DNA polymerase concentration, and the presence of chemicals such as tetramethylammonium chloride (TMAC) or Perfect Match. *See* Charlieu at pages 105-106. Charlieu concludes that "[t]he stringency of PCR is defined by a combination of these factors." *See* Charlieu at page 106. Nowhere is it suggested that RNA can influence 3'-terminal mismatch discrimination. Thus, a person of ordinary skill in the art would not have regarded the difference in terminal mismatch discrimination alluded to in Major as being caused by RNA in bacterial colony lysates.

Finally, Applicants submit that a person of ordinary skill in the art would not have had any motivation to combine Maudru with Major since these references deal with entirely

different, non-analogous assay systems. The Examiner stated that a person of ordinary skill in the art would have been motivated to include a ribonuclease digestion step in the assay of Major because Maudru teaches that "background signal in a similar assay was found to be due to an intrinsic RNA-dependent DNA polymerase activity of the *Taq* DNA polymerase. . ." *See* Office Action at page 8. Applicants respectfully disagree with this assertion.

The assay system of Maudru is concerned with assaying the presence of reverse transcriptase in a sample. The assays are intended to be used to detect the presence of retroviruses for which the sequence of the viral genome is unknown. *See* Maudru, sentence bridging pages 247-248. The assays mentioned in Maudru involve combining a sample suspected of containing a reverse transcriptase with an RNA template, followed by PCR amplification of the cDNA that is produced by the reverse transcriptase. *See* Maudru at page 248, left column. The "background" mentioned in Maudru is DNA that is produced in the PCR step when no reverse transcriptase is present in the sample. (Samples that do not contain reverse transcriptase should not produce any amplified DNA in the PCR step.)

According to Maudru, the background signal is due to the intrinsic RNA-dependent DNA polymerase activity of AmpliTaq, and the background signal can be eliminated by treating the products of the reverse transcription step with RNase prior to the PCR step. A person of ordinary skill in the art would not have regarded the errors in 3'-terminal nucleotide mismatch discrimination mentioned in Major as being caused by RNA-dependent DNA polymerase activity of *Taq* polymerase, especially since errors in 3'-terminal nucleotide mismatch discrimination were known to occur in systems that *lacked RNA*. *See*

discussion above. Thus, a person of ordinary skill in the art would not have had any motivation to combine the RNase treatment step of Maudru with the assay of Major.

In addition, the background signal in Maudru is due to the production of DNA molecules from an RNA template in samples that lack an RT enzyme. This background signal is completely different from and arises from a different source than the "extra minor bands" mentioned in Major which were caused by primer extension in the presence of a 3'-terminal mismatch. Since the "background" mentioned in Maudru is entirely unrelated to the "extra minor bands" mentioned in Major, a person of ordinary skill in the art would have had no motivation to apply the strategy of Maudru for eliminating the background signal (*i.e.*, treating the products of the RT step with RNase) to the assay set forth in Major.

Applicants therefore submit that a person of ordinary skill in the art would have had no motivation to modify or combine Major and/or Maudru. Thus, a *prima facie* case of obviousness has not been established. Applicants respectfully request that this rejection be reconsidered and withdrawn.

Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present

application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Effects of primer–template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies

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ABSTRACT

We investigated the effects of various primer-template mismatches on DNA amplification of an HIV-1 *gag* region by the polymerase chain reaction (PCR). Single internal mismatches had no significant effect on PCR product yield while those at the 3'-terminal base had varied effects. A:G, G:A, and C:C mismatches reduced overall PCR product yield about 100-fold, A:A mismatches about 20-fold. All other 3'-terminal mismatches were efficiently amplified, although the G:G mismatches appeared to be more sensitive to sequence context and dNTP concentrations than other mismatches. It should be noted that mismatches of T with either G, C, or T had a minimal effect on PCR product yield. Double mismatches within the last four bases of a primer-template duplex where one of the mismatches is at the 3' terminal nucleotide, in general, reduced PCR product yield dramatically. The presence of a mismatched T at the 3'-terminus, however, allowed significant amplification even when coupled with an adjacent mismatch. Furthermore, even two mismatched Ts at the 3'-terminus allowed efficient amplification.

INTRODUCTION

Viral genomes, particularly those of RNA viruses and retroviruses, contain multiple base alterations, additions, duplications, and deletions. The variability of these viruses has been attributed to the low fidelity and lack of proofreading functions of the polymerases responsible for their replication (1). In addition, the fidelity of RNA polymerase II which plays a critical role in the retroviral life cycle, and of the reverse transcriptase must be taken into consideration. The repeated rounds of replication required for infection further magnify variability. The role that these viral variants play in the natural history of infection is only beginning to be ascertained and appears to vary with each class of virus.

The polymerase chain reaction (PCR)(2–4) has proven to be a sensitive and specific assay for the detection of retroviral sequences (see for example 5–8). Because of the inherent genetic variability of these viruses, detection by PCR requires the identification of primers that will recognize the viral variants. To ensure the efficient amplification and detection of such viruses, we selected primers that amplify regions of viral genomes that contain either conserved amino acid or nucleic acid sequences. In the former case, the selection of conserved regions encoded by amino acids with minimal codon degeneracy reduces the number of possible oligonucleotides required to prime the region (9). In the latter case, the identification of regions conserved in a large number of sequenced isolates will aid in the selection of primer pairs that will amplify most viral variants (Human Retroviruses and AIDS, 1989, Los Alamos National Laboratory).

The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors, including a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures, b) the effects on duplex stability of mismatched bases and their location, and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. Since single mismatches at or near the terminal 3' base of a primer are known to affect both oligonucleotide stability and efficiency of polymerase extension, they should effect PCR more dramatically than mismatches at other positions (10).

Several investigators have begun to evaluate the effect of single 3'-terminal mismatches (11–15). In this study, we evaluated the effect on PCR of various primer-template mismatches used for amplification of a region of the human immunodeficiency virus type 1 (HIV-1). The importance of HIV detection (5,6,8 16–22) coupled with the heterogeneity of the HIV genomes (23–26), suggested that primers for this virus would serve as an important model for this study.

MATERIALS AND METHODS

HIV Model System

A 130 bp region of *gag* in HIV-1 (NT 1377–1506 of HIVSF2) was amplified by pairing the upstream primer, SK145 or its derivatives (Table I) with the downstream primer SK150 (5' TGCTATGTCACTTCCCCTTGGTTCTCTC). Oligonucleotide SK102 (5' GAGACCATCAATGAGGAAGCTGCAGAATGGGAT) hybridizes to a region within the amplified product and is used as the probe in Southern blot and oligomer hybridization analyses (see below). The oligonucleotides described here also amplify HIV-2.

The templates used in this study were either a recombinant plasmid that harbors an HIV-1 genome, Z6 (gift of C.Y. Ou and G. Schochetman, CDC), or were templates generated by PCR (see below). The sequence of plasmid Z6 at the primer annealing sites was determined by amplification and cloning into M13 of a 300 bp fragment that spans the 130 bp fragment of interest. Sequence analysis shows that SK145 is homologous to Z6 and SK150 differs from plasmid Z6 at a single nucleotide 22 bases from the 3′ end. The single base alteration in SK150 is to more efficiently provide amplification of HIV-2. The sequence of SK150 therefore is a hybrid sequence varying by a single base from the type 1 and type 2 viruses.

Table I. Sequence of SK145 and its Derivatives.

PRIMER DESIGNATION	PRIMER SEQUENCE	POSITION OF BASE ALTERATION
SK145	AGTGGGGGGACATCAAGCAGCCATGCAAAT	NONE
289	----------A	3′
290	----------G	3′
291	----------C	3′
292	----------T-	-1
293	----------G-	-1
294	----------C-	-1
295	----------T--	-2
296	----------G--	-2
297	----------C--	-2
298	----------T---	-3
299	----------G---	-3
300	----------C---	-3

Dashes represent nucleotides that are identical to SK145. Upper case letters denote mismatches relative to SK145.

Oligonucleotides

Two sets of oligonucleotides were synthesized and used for this study. One set of oligonucleotides that differed from SK145 at one of the last 4 bases of the 3′-terminus was used to study the effects of mismatches on amplification (Table I). A comprehensive study of the effects of various primer:template mismatches requires not only primers with base alterations but also templates with various base alterations. We chose to generate templates with the desired change(s) by PCR amplification of plasmid Z6 with a set of 'mutagenic' oligonucleotides. The 'mutagenic' oligonucleotides (SK277 to SK288 and SK312 to SK320) are identical to SK145 at their 5′ ends but have incorporated into their sequence the altered base(s) and are

Table II. Sequence of "Mutagenic" Primers

PRIMER DESIGNATION	PRIMER SEQUENCE	POSITION OF BASE ALTERATION(S)
SK145	AGTGGGGGGACATCAAGCAGCCATGCAAAT	NONE
277	----------Agtta	3′
278	----------Ggtta	3′
279	----------Cgtta	3′
280	----------Ttgtt	-1
281	----------Gtgtt	-1
282	----------Ctgtt	-1
283	----------Tatgt	-2
284	----------Gatgt	-2
285	----------Catgt	-2
286	----------Taatg	-3
287	----------Gaatg	-3
288	----------Caatg	-3
312	----------TCgtta	3′,-1
313	----------CCgtta	3′,-1
314	----------GCgtta	3′,-1
315	----------TAgtta	3′,-1
316	----------CAgtta	3′,-1
317	----------GAgtta	3′,-1
318	----------TGgtta	3′,-1
319	----------CGgtta	3′,-1
320	----------GGgtta	3′,-1

Dashes represent nucleotides that are identical to SK145. Upper case letters denote mismatches relative to SK145. Lower case letters denote bases extended beyond mismatch.

extended by 4 bases beyond the 'mutagenic' base(s) (Table II). We assumed that the 4 additional complementary bases at the 3' end would facilitate extension by *Thermus acquaticus* (*Taq*) DNA polymerase. The predicted Tms for SK145 and SK150 are 83°C and 74°C, respectively, in 0.1M NaCl at a concentration of $1X10^{-6}$M using an algorithm proposed by Breslauer (27,28). The internal G:A mismatch between SK150 and plasmid Z6 should have little effect, if any, on the thermostability of the duplex (29).

The oligonucleotides were synthesized on a Model 8750 DNA synthesizer (Milligen/Biosearch, San Rafael, CA) using long-chain alkylamine controlled pore glass supports and beta-cyanoethyl N,N-diisopropyl phosphoramidites (American Bionetics, Hayward, CA). Standard ancillary reagents and synthesis protocols were used (30,31). The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis which removes contaminating truncated sequences and subsequently desalted by reversed-phase HPLC. Since the oligonucleotides were synthesized 3' to 5', 3' truncated oligonucleotides were not present in the preparations. Base composition analysis was performed to assure that the isolated fragment contained the appropriate number and ratio of nucleosides.

DNA Amplifications

DNAs were amplified in 100 μl reaction volumes with 50 pmoles of each primer, 2 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus Inc.) and either 800, 50, or 6 μM total dNTPs in a buffer containing 10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$. The concentration of primers used was determined by optical density and evaluated by gel electrophoresis.

All amplifications were performed using either plasmid Z6 or PCR-generated products as templates. To generate the base-altered templates, ten thousand copies of Z6 plasmid were amplified in the absence of human genomic DNA. The amount of base altered templates generated by PCR was estimated by comparing the intensities of the ethidium bromide stained product bands to known amounts of marker DNA. Each template was diluted and normalized to the desired concentration before use. Since each PCR-generated product was diluted by at least 10^6 for use as template, contribution of original Z6 molecules to subsequent amplifications is highly unlikely. To determine the effects on PCR of primer-template mismatches, concentrations of approximately 10,000 and 100 copies of either plasmid Z6 or PCR-generated templates were amplified in the presence of 1 μg human placental DNA. Plasmid DNA concentration was determined by optical density at 260nm.

Samples were amplified by 30 repeated cycles on a DNA Thermal Cycler (Perkin-Elmer Cetus, Inc.) using the following parameters: DNA denaturation, 25 sec at 95°C; primer annealing, 25 sec at 55°C; and primer extension, 1 min. at 72°C. For generation of templates with two consecutive mismatches at the 3' end, 'mutagenic' oligonucleotides with 2 consecutive mismatches with plasmid Z6 at positions 5 and 6 from the 3' terminus (SK312 to SK320) were used. In order to achieve more efficient amplifications, a lowering of the annealing temperature to 37°C was necessary. Presumably, these two consecutive mismatches were sufficient to disrupt the stability of the AT-rich 3' end. All amplifications were performed in triplicate and repeated separately with multiple stock solutions.

Detection

The analysis of two different target concentrations and the dramatic differences in the overall yield of the amplification reactions required the use of different detection schemes. Products of high copy target amplifications were analyzed by NuSieve agarose gel electrophoresis and ethidium bromide staining, and confirmed by Southern blot analysis with a 32P end-labeled SK102 probe (specific activity of 1.5−3μCi/pmole) (32). Oligomer hybridization was used to analyze the products of low copy target amplifications (18). As described here, the PCR product yield from any one reaction varies by no more than 50% from sample to sample and run to run. For simplicity, this variability was not incorporated into Table III.

Table III. Relative amplification efficiencies of 3'-terminal mismatches in the presence of 800 μM dNTPs. Product yields were normalized to the perfect matches (1.0).

		Primer 3' Base			
		T	C	G	A
Template 3' Base	T	1.0	1.0	1.0	1.0
	C	1.0	≤.01	1.0	1.0
	G	1.0	1.0	1.0	≤.01
	A	1.0	1.0	≤.01	0.05

RESULTS AND DISCUSSION

Mismatches at the 3' terminus of a primer

The synthesis of oligonucleotides that differ only at the 3'-terminal base, coupled with templates that contain different bases at the corresponding position, provide a system to test the effect of mismatches on PCR. Although amplification of a subset of these templates and primers would provide information on all mismatches, we chose to generate and amplify each template with the four available primers for two reasons. First, we wanted to determine whether the effects of mismatches on PCR were symmetrical. For example, would a G:T (primer:template) mismatch have the same effect on PCR as a T:G mismatch? Second, an intrinsic 3' to 5' exonuclease activity has not been demonstrated for *Taq* polymerase (33,34), therefore, an asymmetric effect of mismatches on PCR may reflect 'context' effects (role of flanking sequences) on the efficiency of extension of a mispaired primer-template.

The results from the 3' mismatch experiments are summarized in Table III. Under the conditions used, and with 800 μM total dNTPs, most mismatches did not significantly affect amplification. In fact, the presence of a T at the 3' end of the primer provided efficient amplification irrespective of the corresponding nucleotide in the template. The mean amplification efficiency per cycle of SK145−150 and these mismatched primer pairs is approximately 85% after 30 cycles. Examination of PCR product after 25 cycles suggested that the efficiency of amplification between 25 and 30 cycles was similar to the amplification efficiency of the earlier cycles. The amplification efficiencies were determined as previously described (3). An A:A (primer:template) mismatch resulted in a 20-fold reduction in overall product yield and A:G, G:A and C:C resulted in approximately a 100-fold reduction. The effects of the mismatches on PCR were symmetrical. For example, both A:G and G:A mismatches were equally detrimental to PCR under these conditions. A representative gel and Southern blot analysis of these amplifications is shown in Figure 1 (brackets 1−3).

Variations in the reaction components and annealing temperatures may affect amplification by mismatched primers. In this study, we examined the effect on amplification of lowering the dNTPs to 50 μM and 6 μM. The results from the

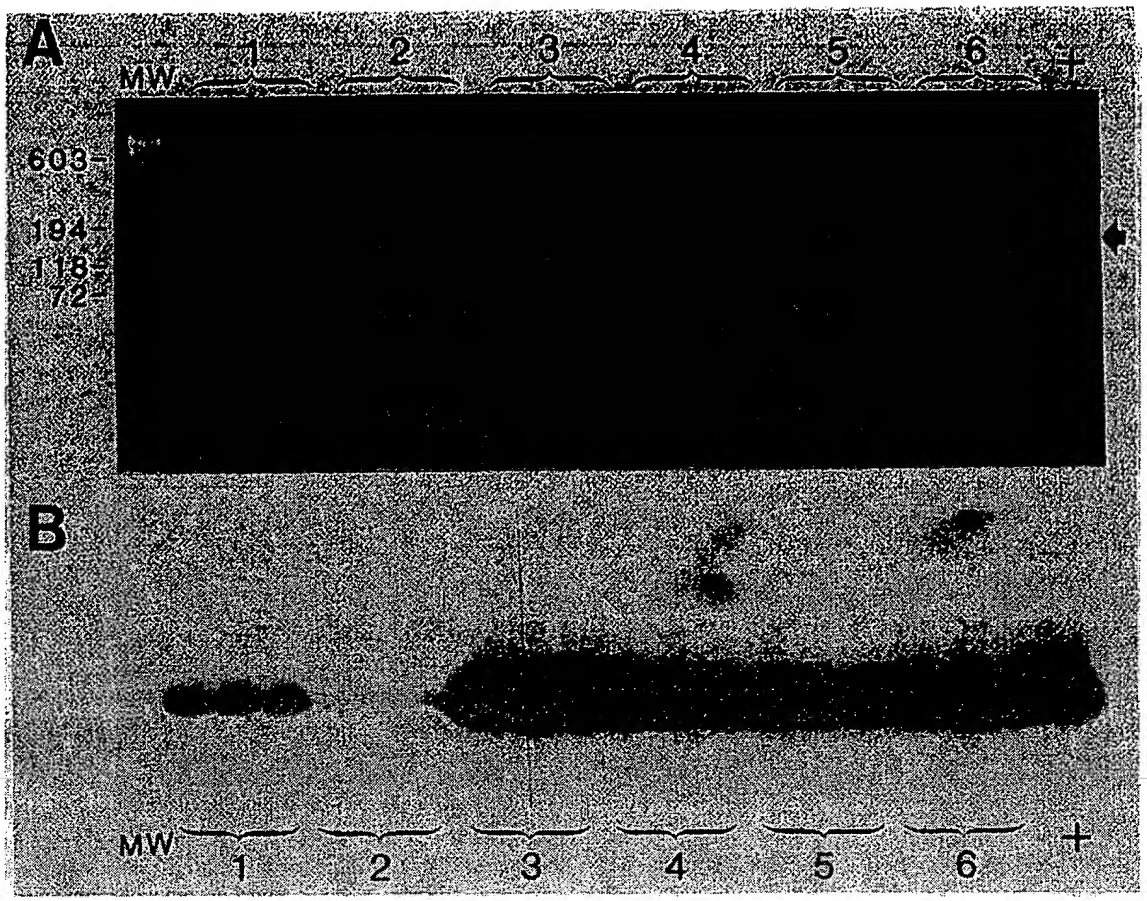

Figure 1. Representative amplifications of plasmid Z6 DNA with primers that were mismatched with template. Samples were amplified in triplicate and visualized on NuSieve agarose gel (Panel A) and analyzed by Southern blot (Panel B). Plasmid Z6 was amplified by coupling SK150 with a primer that had either an A:A, G:A or C:A primer:template mismatch at the terminal 3′ position in 1, 2, and 3, respectively; T:T, G:T or C:T mismatches one nucleotide from the 3′ terminus in 4, 5 and 6, respectively. The positive control (+) represents amplification of plasmid Z6 DNA with a perfectly matched primer. The arrow indicates the position of the product of interest.

amplifications with 50 μM dNTPs were similar to those with 800 μM dNTPs with the exception that the G:G mismatch amplified poorly. In 6 μM dNTPs, only perfectly matched 3′ends were extended with the exception of a T:G mismatch. The overall product yield with this level of dNTPs was at least 10 to 20-fold less than with 800 μM. However, the absence of detectable product may reflect small differences in amplification efficiency rather than complete inhibition of extension. The lower amplification efficiency is more likely a result of lower dNTP concentrations than the higher relative concentration of $MgCl_2$, as this primer pair amplifies efficiently with a broad range of $MgCl_2$.

One of the important factors that affect PCR product yield is the relative efficiency with which the polymerase extends from a mismatched primer-template duplex. Once extension from a mismatched primer occurs, the resultant product and the complement synthesized in subsequent cycles are fully matched with both primers. The molecules with termini defined by both primers accumulate exponentially. On the other hand, products of extensions from the original plasmid template have only one defined terminus and accumulate linearly. Therefore, the contribution of mismatched extension products to total yield, although significant in early cycles, becomes negligible in later cycles. The observed reductions in PCR product reflect dramatic decreases in the efficiency of mismatch extension relative to perfect match extension. If one assumes that mismatch extensions only contribute significantly in the first ten cycles (at which point the mismatch extended templates represent at best 2% of the fully-matched templates) and that the mean efficiency per cycle after cycle ten is 85%, then our studies using 800 μM dNTPs, suggest that an A:A mismatch has an average per cycle efficiency of 50% and the A:G, G:A and C:C mismatches at only an average per cycle efficiency of 16% relative to perfect matches during the first ten cycles. Changes in the reaction conditions such as the concentration of magnesium chloride and annealing temperatures are expected to alter the results described here.

Although the product yields obtained by amplification of a common template with different primers can be directly compared, the products generated from amplification of different templates with common primers may vary somewhat as a result of differences in the amount of PCR-generated template used to seed each reaction. Although the templates were diluted and normalized, the possibility exists that minor differences in product yield merely reflect differences in initial template copy number. Minor differences in template and primer concentrations, however, would not account for the dramatic effects on PCR that

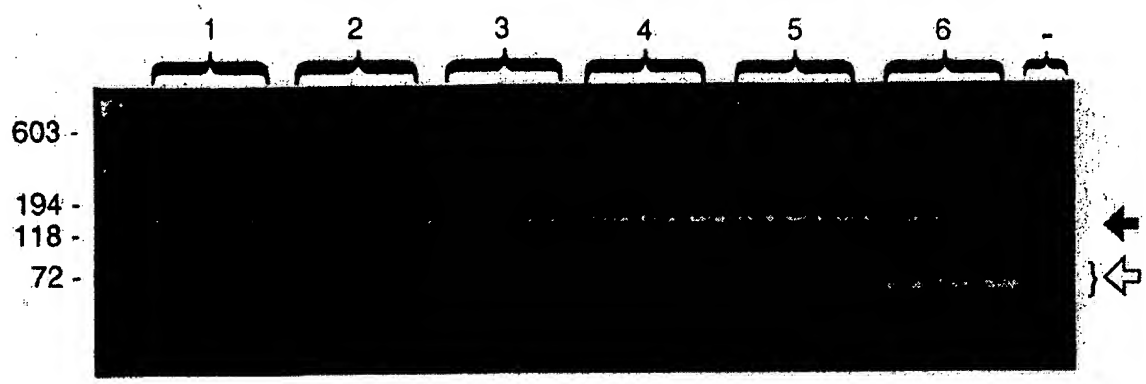

Figure 2. Representative amplifications of plasmid Z6 DNA with primers that differ by a single nucleotide. The plasmid DNA was amplified in triplicate in the presence of 1 ug human placental DNA by coupling SK150 with SK295–300 in (1–6), respectively. Amplification of human placental DNA in the absence of plasmid Z6 is shown in the lane designated (–). The solid arrow shows the position of the desired product; the open arrow shows the positions of the primer-dimers.

were observed with some mismatches. All amplifications were first performed on a high level of target molecules and were repeated on a low level of target. It is expected that effects on PCR yield would be observed in the presence of few targets that may otherwise be masked when amplifying high levels of target molecules. The results from the low target experiments were similar to those obtained from the high target experiments.

Single internal mismatches

In the process of generating templates with altered bases, we demonstrated that a single mismatch 3 residues from the 3′ terminal base of a primer can be efficiently extended without modification of amplification reaction conditions. Similarly, mismatches 1, 2, or 3 bases from the 3′ nucleotide of primers had no apparent affect on overall PCR product yield (for representative amplifications, see figure 1, brackets 4–6). Of particular note are the differences in non-specific amplification products of various molecular weights generated by these reactions. Amplification of the various 'mutated' templates with a common primer pair (SK145–150) resulted in background products that were similar in size. In contrast, amplification of a common template with primers that differ by a single base gave rise to background amplification products that varied dramatically in intensity and size (Figure 2). These data suggest that minor modifications in the primer sequence can dramatically affect the specificity of the amplifications.

In addition, a major product that migrates at approximately the distance expected of the sum of the two primers is often observed in amplification reactions. These products have been cloned and analyzed from several different primer-pair systems (B. Watson, S. Kwok, personal communications). Sequence analysis reveals a fragment bearing the sequence of one of the primers contiguous with the sequence of the complement of the other primer and hence, the product has been termed 'primer-dimer'. Primer-dimers containing a sequence homologous to one of the primers contiguous with its complement have not been identified. It is thought that such a molecule would form a stable hairpin loop structure and would therefore not readily amplify. In some cases, the two primer sequences were separated by 1–10 nucleotides. The mechanism by which these primer:dimers form is not clear. Two alternative explanations can be used to explain these amplification products. First, one of the single stranded primers binds to the polymerase and uses the 3′ end of the second primer as a template for extension. Second, genomic DNA and trace quantities of nucleic acids present in the reactions may contain sequences that are contiguous and similar enough to the primers that they serve as a template to generate these molecules. Given that the downstream primers in these reactions are identical and that the upstream primers differ by only a single base change, it is interesting that amplification of a common template gave rise to not only background products of different sizes and intensities but also primer-dimers of different sizes and intensities (Figure 2).

Multiple mismatches

The effect on PCR of mismatches in a primer:template duplex depends largely on the position and nature of the mismatches. We demonstrated above that some base mismatches (C:T, G:T, T:T, G:G, T:G, T:C, A:C, C:A,) at the 3′-terminus did not have a significant effect on PCR using the conditions described. Although some 3′ mismatches affected amplification, single primer-template mismatches 1 (i.e. penultimate base), 2 or 3 bases from the 3′-nucleotide of a primer did not have a significant affect on PCR product yield. However, when mismatches not involving a T at the 3′ end were coupled with any additional mismatch either 1, 2, or 3 bases from the 3′-nucleotide, PCR product yield was reduced by at least 100-fold (data not shown). However, a T mismatch at the 3′ terminus coupled with an additional mismatch at the penultimate position, resulted in only a 5–10 fold reduction in product yield. Furthermore, the presence of 2 Ts at the 3′ terminus of the primer resulted in overall product yields that were reduced by only 2–5 fold when compared to amplifications by the perfectly matched primers. For these studies, primer pairs SK289–150, SK290–150, SK291–150 or SK292–150 were used to amplify templates generated by amplification of plasmid Z6 with primer pairs SK280–150 through SK288–150 or SK312–150 through SK320–150 (see Tables I and II).

In the generation of templates with two consecutive base alterations, we found that mismatches 5 and 6 residues from the 3′ end had a detrimental effect on amplification when a 55°C annealing temperature was used. However, by lowering the annealing temperature to 37°C, a more efficient amplification was achieved. Because of the AT richness of the 3′ terminus of

these primers, we speculate that the presence of 2 consecutive mismatches 5 and 6 bases from the 3′ terminus sufficiently destabilized the 3′ terminus such that extension by *Taq* DNA polymerase was very inefficient.

The presence of multiple mismatches at least 8 bases from the 3′ terminus of SK145 does not appear to have a significant effect on PCR under the conditions used. A molecular clone of HIVMAL (35) has five mismatches with SK145 and a single G:A mismatch 21 bases from the 3′-terminus with SK150.

```
SK145   AGTGGGGGGACATCAAGCAGCCATGCAAAT
HIVMAL  ---T--A-----C--G-----T--------
```

One would predict that the Tm of SK145 on the HIVMAL template might be at least 10°C lower. Despite these mismatches, HIVMAL was efficiently amplified under the conditions used. However, when the annealing temperature was raised to 60°C, PCR product yield was significantly reduced, and at 65°C, amplification was not detected. These results underscore the importance of using less stringent annealing temperatures for the amplification and detection of highly variable targets.

CONCLUSIONS

Several conclusions can be drawn from this study. Certain mismatches at the terminal 3′ position (ie. T:C, T:G, T:T, G:G, and A:C) appear to amplify as efficiently in PCR as the fully complementary primer-template duplex, under these conditions, while an A:A mismatch moderately reduced PCR amplification efficiency per cycle, and A:G and C:C mismatches dramatically reduced PCR amplification efficiency. Although G:G mismatch amplified efficiently at 800 μM dNTPs, the product yield in 50 μM dNTPs was dramatically reduced. Whereas some 3′-terminal mismatches were poorly extended, single base mismatches between the primer and template either one, two or three bases from the 3′ nucleotide of the primer can be extended without a significant effect on overall product yield. However, when coupled with an additional mismatch within the last four bases, overall PCR product yield from a 3′ terminal mismatched primer is drastically reduced. In contrast, oligonucleotides with a T mismatch at the 3′ terminus when coupled with an additional mismatch at the penultimate position served efficiently as primer for amplification. Further, the presence of 2 Ts at the 3′ terminus enabled amplification of templates with mismatches at both positions irrespective of the nucleotides involved.

A priori, the thermodynamic stability of base mismatches cannot explain the results observed here. The efficiency at which polymerase extends from a mismatched base pair depends on a number of complex interactions. First, the overall stability of the primer-template may determine the likelihood that polymerase binds to a duplex. Second, extension by polymerase probably reflects recognition of the base stacking, hydrogen bonding, and overall steric structure of the terminus to be extended. These factors may themselves interact since hydrogen bonds may facilitate stacking by bringing the bases in proximity and vice versa. Although numerous studies have begun to describe the calculated and measured stabilities of the various mismatches alone and relative to sequence context (10,36), the simplest interpretation of our data is that purine-purine mismatches do not extend efficiently while pyrimidine-pyrimidine and purine-pyrimidine mismatches do extend efficiently under these conditions. The G:G and C:C mismatches serve as exceptions to these conclusions. Perhaps the stacking forces of the G residue with the penultimate base and the hydrogen bonded structure proposed by Abou-ela, et al (29) play a role in efficient extension of this mismatch by polymerase. On the other hand, the proposed sugar-phosphate constrained structure with only one hydrogen bond for a C:C mismatch may disrupt the 3′-terminal structure to the extent that efficient extension is not possible (29). Extension of the primer-template duplex from a G:T mismatch perhaps would have been expected since it contributes neither a stabilizing nor destabilizing influence to the nucleic acid duplex (37). The efficient extension of a T:C or T:T mismatch was not, however, expected. Recent studies by M.Goodman and colleagues on the relative extension efficiencies of mismatched termini by AMV reverse transcriptase and DNA polymerase alpha are in general agreement with our results (38). The extent to which one can extrapolate our data to other primer pair-template systems will require additional studies. The extent to which the three contiguous A's near the three terminus contributes to these results is unclear. However, our preliminary experiments with other HIV and HTLV primer pairs support the general applicability of these observations. Included in these experiments are data suggesting that the efficiency of extension of a G:G mismatch may be dependent on sequence context as well as on the concentration of deoxynucleoside triphosphates.

Several investigators have begun to explore the effects of mismatches on the amplification of cellular sequences. A direct comparison of our results with those previously published is difficult due to differences in reaction conditions (buffer, primer concentration, annealing temperature), target copy number, primer length and sequence context. In some studies, the primers were short (12 – 16 bases) and therefore a 3′ mismatch may have more dramatically affected overall primer:template duplex stability as well as the ability for the polymerase to catalyze extension.

Our results are in agreement with those of Ehlen et al. (13) where the amplifications were carried out under similar reaction conditions and with primers of similar length. Ehlen et al. (13) demonstrated that at 200 μM dNTPs, a C:T mismatch can be extended where as at 2 μM dNTPs C:T, A:C and C:C mismatches were not extended. Using similar conditions but with primers that were 16 bases long, Nichols et al. (12) also showed that an A:A mismatch was not extended. Wu et al. (11), again under similar conditions but with primers that were 14 bases in length, presented evidence that A:A and T:T mismatches were not extended at an annealing temperature of 55°C but were extended at annealing temperatures of 44°C and 50°C. The extension of the A:A mismatch at the lower annealing temperature is contrary to our finding. The studies of Okayama et al. (15) were also carried out under similar conditions but with shorter primers. Their results are contrary to all other studies in that 10 of the 12 possible mismatches tested prevented significant amplification; the A:G and C:T mismatched were not evaluated.

Newton et al. (14) demonstrated the use of 3′-terminal mismatched primers for allele-specific amplifications. In their study, G:T, T:G, A:C, and C:A, mismatches were extended by *Taq* DNA polymerase whereas A:A, T:T, and C:T mismatches were refractory to extension. The C:T and T:T results are contrary to our findings and may reflect the substantial differences in the reaction conditions. Consistent with Newton et al., we found that single internal mismatches did not significantly affect

PCR product yield. Furthermore, the placement of an additional internal mismatch to extendable 3'-terminal mismatches proved refractory to amplification.

Implications of this study extend to multiple areas. First, primers for nucleic acid templates from pathogens known to vary in sequence would benefit from a single T or perhaps double T at the extreme 3' end so as not to obviate amplification because of mismatched bases at 3' end. The HIV-1 model system used in this report is of particular importance given the extensive heterogeneity within and among infected individuals. Ideally, the primers used in a diagnostic assay for this virus should not only have the desired sensitivity and specificity on contemporary isolates but should also be engineered to accommodate a degree of divergence expected in future isolates. The observations noted in this study may contribute to the design of primers for the detection of other variable pathogenic viruses such as the human papilloma viruses and RNA viruses. Second, this information will aid in the design of primers used in the search for additional members of known virus groups (7,9). For example, the design of primers with T rather than A or G at the 3' terminus may increase the likelihood of extension.

On the other hand, there are applications in which primers need to be designed for allele-specific amplifications. The presence of a 3' terminal A:G or C:C mismatch, and to a lesser extent an A:A mismatch, should bias the amplification to the desired targets. In addition, alterations in the concentration of reaction components (13) and annealing temperature should also effect extension from mismatched primers and therefore should also be exploited.

Finally, we expect that studies similar to those described here will begin to address the specific protein-nucleic acid interactions of polymerases and their substrates so that we may better understand substrate binding and catalysis by this family of enzymes.

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Chapter 12

DISTINCTION BETWEEN ALMOST-IDENTICAL DNA SEQUENCES BY POLYMERASE CHAIN REACTION

Jean-Paul Charlieu

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I. INTRODUCTION

Two DNA fragments sometime present very strong sequence homology. For example, the alleles of a gene can differ at only one position, when a point mutation occurs. Several techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), and hybridization with allele-specific oligonucleotides (ASO) allow discrimination between almost-identical DNA sequences.

It has been shown recently[1] that PCR can also be used to distinguish DNA variants in the HIV genome. Based on this study, PCR conditions were developed to distinguish the alpha-satellite subfamilies of chromosomes 13 and 21.[2] Alpha-satellite is a family of tandemly repeated DNA sequences present at the centromeric region of all human chromosomes.[3] Slight variations in the nucleotide sequence of the ~171-bp basic motif and in the distribution of restriction sites define higher order repeats that are specific for one or a few chromosomes.[4] The subfamilies from chromosomes 13 and 21 are almost identical, however, as they share 99.7% homology (2 out of 680 bp).[5] Hybridization experiments performed with a probe from one of these chromosomes reveal the alpha-satellite fragments from both.[6]

A membrane-bound PCR[7] approach allowing the chromosomal origin (13 or 21) of alpha-satellite fragments detected by Southern blot hybridization is presented here. The experimental parameters that influence the PCR specificity also have implications for other purposes such as the detection of point mutations and the discrimination between the alleles of these models.

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II. MATERIALS AND METHODS

A. PRIMERS

Based on the nucleotide sequence of alpha-satellite subfamilies from chromosomes 13 and 21,[5] two pairs of primers (13A + 13B and 21A + 21B) were designed:

13A: 5′ TGATGTGTGTACCCAGCT 3′
13B: 5′ GCTATCCAAATATCCACT 3′
21A: 5′ TGATGTGTGTACCCAGCC 3′
21B: 5′ GCTATCCAAATATCCACC 3′

Each primer carries a chromosome-specific nucleotide at its 3′ end. They were resuspended in sterile distilled water to obtain stock solutions at 20 μ*M*.

B. BUFFERS AND REAGENTS

Distilled water was filtered through 0.22-μm filters and autoclaved at 120°C for 20 min before use. Small aliquots (1 ml) were reserved for PCR assays. Bovine Serum Albumine (BSA) was purchased from Boehringer Mannheim, diluted with water to 0.5 mg · ml^{-1}, and stored as 0.5-ml aliquots at –20°C. *Taq* DNA polymerase buffer 10 × concentrated was supplied by Promega: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.8 at 25°C, 15 m*M* $MgCl_2$, 1% Triton X-100. Nucleotides, from Boehringer Mannheim, were separately resuspended in water to obtain stock solutions at 50 m*M*. Working solution (10 × concentrated) consists of a mix of the four dNTPs at 0.025 m*M* each. Diluted dNTPs are quite unstable and should be kept as small aliquots (20 μl) at –20°C. *Taq* DNA polymerase was from Promega, and Perfect Match polymerase enhancer was from Stratagene.

C. DNA TEMPLATES

The following somatic hybrids containing the specified human chromosome(s) in parentheses in a rodent genomic background were used: WA17 (human chromosome 21) was obtained from Dr. Devine;[8] HY124VT4, HY73DMT3 (both with human chromosome 21 in a hamster genomic background), RJ387.58T1, HY25T1 (both containing human chromosome 13), RJ387.91CT8 (chromosome 2), and HY129T14 (chromosome 14) were kindly provided by Dr. M. Rocchi; BCHE (human chromosomes 3, 4, 6, 8, 9, 10, 13, 14, 15, 16, 18, 19, Xp^+) and C35B2 (chromosome 11) were from Dr. N'Guyen Van Cong.

Cells were collected and washed twice with phosphate saline buffer (PBS) and lysed with 200 μg·ml^{-1} proteinase K (Appligène) in 0.5 *M* EDTA; 1% L-lauryl sarcosyl, pH 8.5 for 48 h at 50°C. The DNA was then purified by phenol extraction and ethanol precipitation according to standard methods.[9]

DNA was denatured in 0.5 *M* NaOH, 1.5 *M* NaCl for 5 min at room temperature and dotted onto nylon membrane (hybond N, Amersham). When dried, DNA was cross-linked to the membrane with UV light for 5 min. Small pieces (~1 × 1 mm) carrying ~100 ng of DNA were cut to obtain membrane-bound DNA templates for PCR.

Human genomic DNA was prepared embedded in low-melting agarose from blood or cultured cells as previously described.[6] Restriction enzyme hydrolysis, separation of DNA fragments by pulsed field gel electrophoresis (PFGE), Southern blotting and hybridization with the alpha-satellite probe α-RI 680, 368[5] from human chromosome 21 were performed as described[6] except that hybond N (Amersham) nylon membrane was used. After blotting, the filter was dried in an oven at 80°C and the DNA was cross-linked to the membrane with UV light (5 min on a UV table).

Pieces of membrane carrying the alpha-satellite fragments detected by hybridization were used as templates in PCR experiments.

D. PCR CONDITIONS

PCR experiments were performed in a PREM thermocycler as follows:

1. Prepare the sample mix (for 1 sample):

H_2O	9 µl
Taq DNA polymerase buffer 10 × concentrated	2 µl
Primer A, 20 µ*M*	1 µl
Primer B, 20 µ*M*	1 µl
BSA, 0.5 mg·ml^{-1}	2 µl

   Multiply these quantities by the number of PCR samples.

2. Distribute 15 µl of the sample mix in 0.5-ml reaction tubes, and add the DNA template (100 ng) and one drop of mineral oil to prevent evaporation.

3. Prepare the "enzyme mix" (for one sample):

H_2O	2.3 µl
Taq DNA polymerase (Promega), 5 U·µl^{-1}	0.5 µl
dNTPs 10 × concentrated	2.0 µl
Perfect Match polymerase enhancer, 1 U·µl^{-1}	0.2 µl

   Multiply by the number of samples and keep on ice.

4. Program the thermocycler as follows:

   1. 95°C, 5 min
   2. 59°C, 1 s
   3. Suspend the program
   4. 92°C, 5 s
   5. 59°C, 30 s
   6. Repeat from step 4, 40 times
   7. 72°C, 5 min

5. Place the tubes in the thermocycler and run the program. When the program is suspended at the annealing temperature after the initial denaturation, add 5 µl of "enzyme mix" to each reaction tube and continue the program.

For membrane-bound PCR, each piece of nylon filter carrying the DNA template was washed in 10 ml of H_2O for 30 min at 65°C and saturated in 200 µl of 1 × *Taq* DNA polymerase buffer containing 0.5 mg·ml^{-1} of BSA for 15 min at 65°C. The PCR conditions were the same except that 60 cycles were performed.

E. ANALYSIS OF PCR PRODUCTS

PCR products were analyzed in a 10% polyacrylamide gel prepared in TBE 0.5 × from a stock solution 38% acrylamide to 2% bisacrylamide. After 3 h of electrophoresis at 150 V, the DNA was stained by soaking the gel in an ethidium bromide solution (0.5 µg·ml^{-1}) for 30 min and visualized with UV light.

III. RESULTS

The PCR conditions allowing discrimination between the alpha-satellite subfamilies from chromosomes 13 and 21 were developed using the DNA from somatic hybrids containing one of these two chromosomes. A DNA fragment of the expected size (98 bp) was amplified with the two pairs of primers 13A + 13B and 21A + 21B. The nature of this DNA fragment was checked by Southern blot hybridization with the alpha-satellite probe used in this study (not shown).

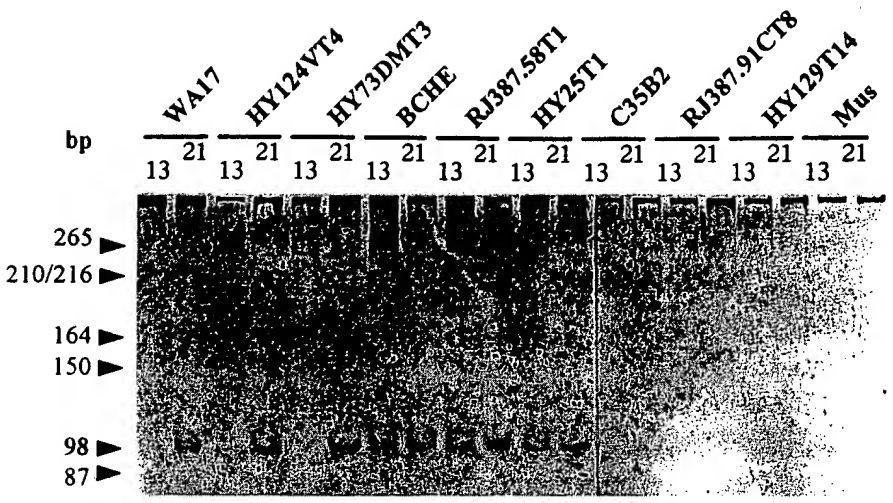

Figure 1. Membrane bound PCR with dot blots carrying the DNA from somatic hybrids noted at the top of the lanes. Numbers 13 and 21 indicate which pair of primers was used. 10 µl of the PCR products were loaded in a 10% polyacrylamide gel. The DNA was revealed by ethidium bromide staining and UV detection. (From Charlieu, J.-P., Murgue, B., Laurent, A.-M., Marçais, B., Bellis, M., and Roizès, G., *Genomics*, 14, 515, 1992. With permission.)

Membrane-bound PCR conditions were tested with dot blots carrying the DNA from the same somatic hybrids (Figure 1).

As for the experiment described above, the 98-bp amplified fragment was obtained with the chromosome 13-specific pair of primers (13A + 13B) only when using somatic hybrids containing chromosome 13, whereas the chromosome 21-specific pair of primers (21A + 21B) allowed the amplification of this DNA fragment in both chromosome 21- and 13-containing hybrids. The negative controls showed that this PCR product does not originate from the rodent genomic background or human chromosomes other than 13 and 21.

In order to test the possibility of determining the chromosomal origin of alpha-satellite fragments revealed by Southern blot hybridization, a CEPH (Centre d'Étude du Polymorphisme Humain, Paris) was used. Genomic DNA prepared in agarose plugs was digested with Bam HI, and the fragments were separated by PFGE. After hybridization and autoradiography, one band characterizing each allele determined by segregation analysis (A1, A2, B1, and B2 from the mother and C1, C2, D1, and D2 from the father)[6] was picked up and used in membrane-bound PCR (Figure 2). Alleles A1, A2, D1, and D2 were found to originate from chromosome 13, and alleles B1, B2, C1, and C2 from chromosome 21.

When analyzed by PFGE, the alpha-satellite Bam HI fragments were found to be quite variable in size. In addition, these alleles were found to segregate in a Mendelian fashion.[6] Alpha-satellite can therefore define a very informative centromeric marker when it is possible to distinguish by PCR the fragments from chromosomes 13 and 21.

IV. DISCUSSION

Annealing temperature is generally the main parameter considered for PCR specificity. In fact, several other parameters should be taken into account to obtain a specific PCR amplification. These are presented in this discussion, based on the example of PCR amplification of alpha-satellite subfamilies from chromosomes 13 and 21. The given values should be considered only as indications for the study of other models for which very stringent PCR conditions are needed.

It has been shown[1,10] that PCR amplification can be performed using primers containing up to 50% of mismatching nucleotides, but there is an absolute requirement for the correct base

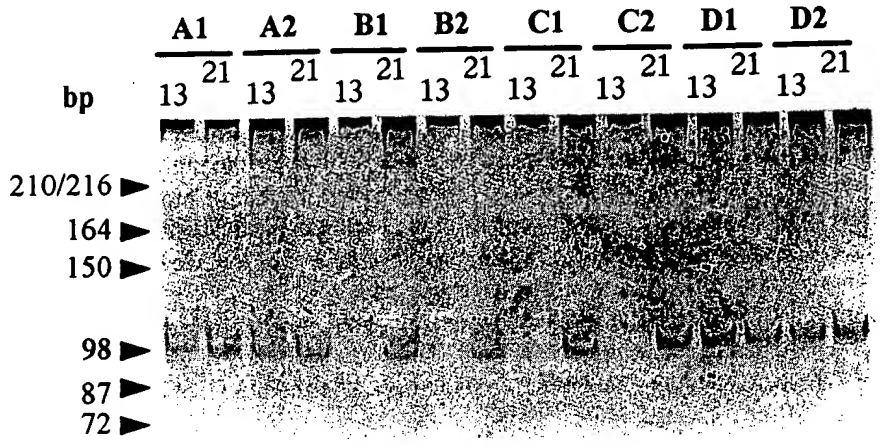

Figure 2. Membrane-bound PCR was performed with alpha-satellite primers on pieces of a Southern blot carrying the alpha-satellite Bam HI fragments revealed by hybridization. The PCR products, obtained with PCR conditions identical to those described in Figure 1, were analyzed in a 10% polyacrylamide gel. The CEPH family K1418 was used in this experiment. (From Charlieu, J.-P., Murgue, B., Laurent, A.-M., Marçais, B., Bellis, M., and Roizès, G., *Genomics*, 14, 515, 1992. With permission.)

pairing of their 3′ end. Thus, the main rule for the design of PCR primers allowing the discrimination between homologous DNA sequences is the *3′ position* of the mismatching nucleotide. In addition, Kwok et al.[1] have shown that all base mispairings do not work with the same efficiency. In particular, they have found that the T:G mismatch has no effect on PCR specificity. In the primary sequence of alpha-satellite DNA from chromosomes 13 and 21, the only differences are T-to-C changes, however.[5] The T:G mismatch (T on 13A and 13B primers and G on the chromosome 21 alpha-satellite DNA template) was found in this study to allow the correct discrimination between these two alpha-satellite DNA sequences: the 98-bp fragment was amplified with the pair of primers 13A + 13B only with the alpha-satellite template from chromosome 13. Thus, there might not be absolute rules in the nature of the mispairing nucleotide but this may depend on the model studied. Amplification of the 98-bp fragment from chromosome 13 with the chromosome 21-specific pair of primers could indicate either that the C:A mismatch (C on the 21A and 21B primers and A on the chromosome 13 alpha-satellite DNA sequence) does not affect PCR specificity or that the alpha-satellite subfamily of chromosome 21 is also present on chromosome 13.

The nucleotide concentration can also determine PCR specificity. In our laboratory, a "standard" PCR sample contains 0.2 m*M* of each dNTP. In these conditions, however, a 98-bp fragment was amplified in all cases with both chromosome 13 and 21 alpha-satellite-specific pairs of primers. Correct specificity was obtained by decreasing the nucleotide concentration to 2.5×10^{-3} m*M*.

$MgCl_2$ concentration also influences the PCR. We found that 1.5 m*M* was a good concentration for our system. Promega now provides *Taq* DNA polymerase buffer without $MgCl_2$, which can be added to give the desired concentration of this salt.

Another parameter affecting PCR specificity is the *Taq* DNA polymerase concentration. With the enzyme used, 2.5 U was the limit required to obtain reproducible and specific PCR amplification. We have also tested the thermostable DNA polymerase from Thermus flavis (Tfl polymerase), and we have found that the 98-bp alpha-satellite DNA fragment can be obtained with 1 or 0.5 U of enzyme, but results were not reproducible. If no specific amplification occurs in a given model, it is advisable to decrease the enzyme concentration or to test other enzymes.

The addition of chemicals such as tetramethylammonium chloride (TMAC)[11] or Perfect Match polymerase enhancer (Strategene) can also make it possible to obtain a specific

amplification. TMAC is generally used at 10^{-4} to 10^{-5} *M*. We have found that these conditions are too stringent, and we could not find the correct concentration for our system. The manufacturer recommands addition of 1 U of Perfect Match to the PCR sample. Again, we found the conditions too stringent for our case, and 0.2 U was sufficient. We recommend addition of Perfect Match with the enzyme and the dNTPs *after* the initial denaturation step in hot-start PCR. This product seems to be inactivated when submitted to a high temperature for a long time. We have also observed that non-specific amplification is reduced in these conditions. The mix of TMAC and Perfect Match can also be tried, but we found that they have a very strong effect on PCR when used together.

The stringency of the PCR is defined by a combination of these factors. We have described the conditions that work for our primers and in our hands. The study of other models or of our model in another laboratory and/or with another PCR machine will probably need some adjustments of the indicated values.

For membrane-bound PCR, we have tested several nylon supports. We have found that positively charged membranes are not convenient for our purpose. It seems that they have an extremely high capacity to capture the DNA and that, if amplification occurs, the PCR products are not released in the medium and cannot be detected. All uncharged nylon membranes we have tested present good qualities for membrane-bound PCR.

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